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Ontogenic development of T and B cells and non-lymphoid cells in the white pulp of human spleen

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SUMMARY

The ontogenic development of lymphoid and non-lymphoid cells in human splenic white pulp was studied histologically with immunoperoxidase technique, together with that of lymphoid cells from fetal liver, bone marrow and thymus by membrane immunofluorescence assay. The primitive white pulp, which appeared as small accumulations of lymphocytes around arterioles at 14 weeks of gestation (g.w.), was mainly composed of B1 antigen-positive B cells. After the appearance of follicular structure accompanied by follicular dendritic cells (FDC) stained with anti-DRC1 antibody at 26 g.w., these perivascular structures of B cells were located in the periphery of the white pulp areas. A large number of B cells composing the perivascular structure had surface IgM (sIgM) and IgD (sIgD) from the earliest stage (14 g.w.), although this type of B cell with mature phenotype was seldom observed in fetal liver or bone marrow at this stage. It was suggested that the spleen is an important site for B-cell maturation from sIg-negative B cells observed in 10–14 g.w. fetal liver, and that FDC are not involved in this development of B cells. The organization of 9.6 antigen-positive T cells around arterioles developed 4 weeks later than that of B cells, at 18 g.w., although 11 g.w. fetal thymocytes already showed a phenotype very similar to that of infants. Interdigitating reticulum cells (IDC) stained with anti-S-100 protein serum appeared from 14 g.w. before the T-cell organization, suggesting that IDC may play an essential role in the homing of T cells.

INTRODUCTION

The ontogenic development of lymphoid tissues has been studied in several species, including man (Friedberg & Weissman, 1974; Asma *et al.*, 1977; Gathing, Lawton & Cooper, 1977; Eikelenboom *et al.*, 1979; Hayward, 1981; Toivanen *et al.*, 1981). Since it is now possible to analyse the differentiation of human lymphocytes with an array of monoclonal antibodies (Reinherz *et al.*, 1980; Bernard, Boumsell & Hill, 1984), several studies on the development of B and T cells by analysis of suspension cells have already been reported (Brashem *et al.*, 1982; Asma, Van Den Bergh & Vossen, 1983; Rosenthal *et al.*, 1983; Kamps & Cooper, 1984). It has been shown that characteristic types of non-lymphoid cells are located in both B and T areas of lymphoid tissues. Follicular dendritic cells (FDC) (Nossal *et al.*, 1968; Hanna & Szakal, 1968) present in lymphatic follicles were reported to act as antigen-presenting cells by trapping antigens, usually in the form of antigen-antibody complexes (Klaus *et al.*, 1980; Mandel *et al.*, 1980). For the T-cell areas, the corresponding cell type is interdigitating reticulum cells (IDC) (Veldman & Kaiserling, 1980; Veerman, 1974), the function of which is not yet elucidated, but Langerhans cells in the epidermis, which have a close morphological similarity to IDC and also in the reactivity with antibody against S-100 protein (Cocchia, Michetti & Donato, 1981; Takahashi *et al.*, 1984), are known to stimulate allogeneic T cells in mixed lymphocyte culture (Stingl *et al.*, 1978) and are considered to be antigen-presenting cells in contact dermatitis (Silberberg-Sinakin *et al.*, 1980). It is, therefore, important to analyse the relationship between these non-lymphoid cells and lymphocytes in tissue sections, in addition to analysing surface phenotype and function *in vitro*.

In the present study, the morphogenesis of human splenic white pulp is analysed with various antibodies, with regard not only to lymphocytes, but also to non-lymphoid cells. The antigenic phenotype of lymphocytes from primary lymphoid tissues is also studied in relation to spleen development.

Abbreviations: FDC, follicular dendritic cells; g.w., gestational week; IDC, interdigitating reticulum cells; PALS, periarterial lymphatic sheath; sIg, surface immunoglobulin.

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MATERIALS AND METHODS

Fetal tissues

Twenty-nine human fetuses from 8 to 20 weeks of gestation (gestational week, g.w.) were obtained by interruption on non-medical grounds. Fetuses older than 20 g.w. were aborted because they suffered from anencephalus, and the permission to use these specimens for the present investigation was given by their parents. The heel-to-toe measurement of Robboy (Robboy, Taguchi & Cunha, 1982) was used to standerdize gestational age.

Small tissue blocks of fetal spleen or other lymphoid tissues sliced 2–3 mm thick were fixed in periodate–lysine–2% paraformaldehyde (PLP) (McLean & Nakane, 1974) at pH 7·2 for 4 hr at 4°, washed in increasing concentrations of sucrose in 0·05 M phosphate-buffered saline (PBS), embedded in OCT compound (Ames, Elkhart, IN), quickly frozen in dry ice–acetone, and stored at −70°.

Immunoperoxidase staining for light and electron microscopy

Six-μm frozen sections were picked up on slides and air-dried for 30 min, washed in 0·01 M PBS, pH 7·4, and then stained by immunoperoxidase technique. For light microscopic observation with mouse monoclonal antibodies (Table 1), avidin–biotin–peroxidase complex (ABC) method (Hsu, Raine & Fanger, 1981) using Vectastain ABC Kit, PK-4002 (Vector, Burlingame, CA) was used. Following preincubation with 0·5% normal horse serum for 20 min at room temperature (RT), they were covered with the adequately diluted antibodies (Table 1) and incubated in a moist chamber overnight at 4°. After washing in cold PBS, sections were incubated with biotin-labelled horse anti-mouse IgG (1:400) for 30 min at RT, washed thoroughly in cold PBS, and then ABC was applied for 45 min at RT. They were then washed and stained for peroxidase activity with 3,3′-diaminobenzidine-tetrahydrochloride (DAB) (Wako, Osaka, Japan) 0·2 mg/ml Tris-HCl buffer, pH 7·6, containing 0·003% H_2O_2.

In the case of rabbit antisera for staining (Table 1), the sections were treated with 2·5% normal swine serum before incubation with the first antibody, and then reacted with peroxidase-labelled swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) diluted 1:40 for 30 min at RT. They were further reacted for peroxidase activity with DAB as described above.

For electron microscopic observation, the two-step immunoperoxidase method was employed. Peroxidase-labelled $F(ab')_2$ rabbit anti-mouse IgG+A+M (Zymed, Burlingame, CA) was applied as the second antibody for mouse monoclonal antibodies, or peroxidase-labelled swine anti-rabbit IgG for rabbit antisera. Sections were fixed with 1% glutaraldehyde–0·05 M phosphate buffer (PB), pH 7·4, before DAB reaction, and fixed again with 2% osmium-tetraoxide. After washing in distilled water, they were stained with saturated uranyl acetate, dehydrated and embedded in Epon mixture as previously described (Namikawa *et al.*, 1983).

Immunofluorescence assay of suspension cells

Fetal bone marrow, liver and thymus were minced in RPMI-1640 medium containing 10% fetal calf serum (FCS). Mononuclear cells were isolated by Ficoll–Paque (Pharmacia, Uppsala, Sweden) sedimentation and the cell composition was examined morphologically by May–Grünwald–Giemsa staining of cytocentrifuged spreads. HL-1 antibody was included to estimate the contamination by myelocyte–monocyte lineage cells

Table 1. Mouse monoclonal antibody and conventional rabbit antiserum

	MW $\times 10^{-3}$	Ig class	Serological reactivity	Working dilution	Reference
Mouse monoclonal antibody					
Tp40	40	IgG1	Thymocyte + peripheral T cell	1:400	Tsuge *et al.*, 1984
Tp120	120	IgG2a	Medullary thymocyte + peripheral T cell	1:400	Tsuge *et al.*, 1984
9.6	50	IgG2b	E-rosette receptor (pan T)	1:100	Kamoun *et al.*, 1981
OKT3	19	IgG2a	Medullary thymocyte + peripheral T cell	1:0500	Reinherz *et al.*, 1980
OKT6	12,49	IgG1	Cortical thymocyte	1:100	Reinherz *et al.*, 1980
OKT4	56	IgG1	Thymocyte + helper T cell	1:100	Reinherz *et al.*, 1980
OKT8	32	IgG1	Thymocyte + cytotoxic-suppressor T cell	1:100	Reinherz *et al.*, 1980
B1	35	IgG2a	B cell (pan B)	1:400	Stashenko *et al.*, 1980
NL-12	28,32	IgG2a	HLA-DR antigen	1:400	Ueda *et al.*, 1982
HL-1	ND*	IgM	Myelocyte–monocyte lineage cells	1:400	Namikawa *et al.*, 1983
DRC1	ND	IgM	FDC	1:100	Naiem *et al.*, 1983
Conventional rabbit antiserum					
IgM			Human IgM heavy chain	1:1000	
IgD			Human IgD heavy chain	1:1000	
S-100	21		IDC (S-100b protein)	1:500	Hidaka *et al.*, 1983

* ND, not determined.

(Namikawa *et al.*, 1983). The specimens with cell viability of more than 95% determined by trypan blue dye exclusion were analysed for the surface phenotype by membrane immunofluorescence as described previously (Tsuge *et al.*, 1984). The percentage positive cells were enumerated after reading at least 200 cells stained.

RESULTS

Organization of B and T cells in developing white pulp studied by immunoperoxidase staining

Spleen consisted of immature mesenchymal cells at 12 g.w., and lymphocytes were seldom observed. Only a few clusters of lymphocytes were recognized in a routine histological preparation of the 14 g.w. fetal spleen stained with hematoxylin–eosin, but the immunoperoxidase staining revealed small accumulations of B1 antigen-positive B lymphocytes around arterioles (Fig. 1a), which became progressively prominent in the succeeding weeks (Fig. 2a). As early as 14 g.w., most of these B lymphocytes had surface IgM (sIgM), and a large proportion also had sIgD. T lymphocytes which react with 9·6, Tp40 and Tp120 antibodies were already found at 14 g.w., but they were scattered throughout the spleen without forming recognizable clusters (Fig. 1b). It was not until 18 g.w. that T cells began to form a cuff around the arterioles (Fig. 2b).

In the spleen of the 26 g.w. fetus, B lymphocytes were found to form the primary follicles, in addition to the perivascular accumulations (Fig. 3a) which were located peripherally to the white pulp area. The cuff of T cells became more prominent in 26 g.w. fetal splenic white pulp, showing a well-organized structure around central arterioles in the form of a periarterial lymphatic sheath (PALS) (Fig. 3b). Almost all T cells constituting this cuff were also positive for Tp40 and Tp120 antigens. As for T-cell subset antigens, there were more T4-positive cells than T8-positive cells. Thus, the basic structure of the white pulp composed of PALS with surrounding B areas and follicles as observed in the adult form was established at 26 g.w.

Appearance of non-lymphoid cells in developing white pulp studied by immunoperoxidase staining

Although B lymphocytes were organized as perivascular accumulations from the early stage of development, FDC defined

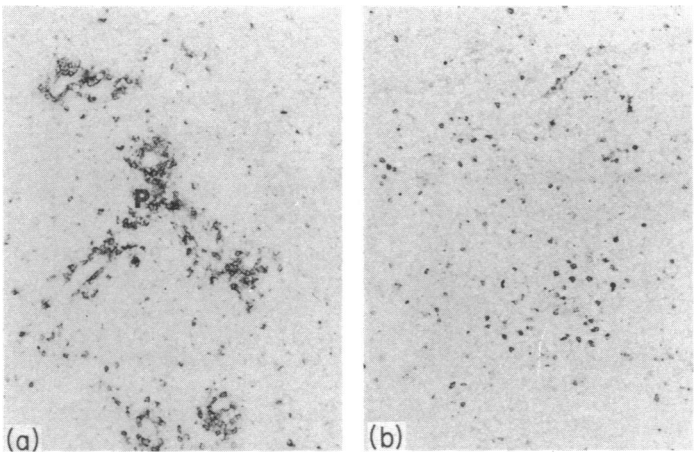

Figure 1. Spleen of 16 g.w. fetus. (a) Staining with B1 antibody shows prominent perivascular accumulations of B cells (P). (b) T cells stained with Tp40 antibody do not form recognizable clusters. (Magnification × 80.)

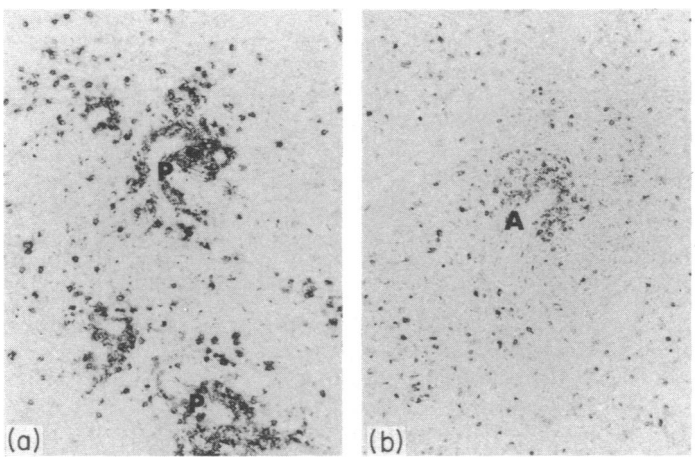

Figure 2. Spleen of 18 g.w. fetus. (a) Perivascular accumulations of B1-positive cells (P) become more prominent. (b) T cells with Tp40 antigen begin to accumulate around central arterioles (A). (Magnification × 80.)

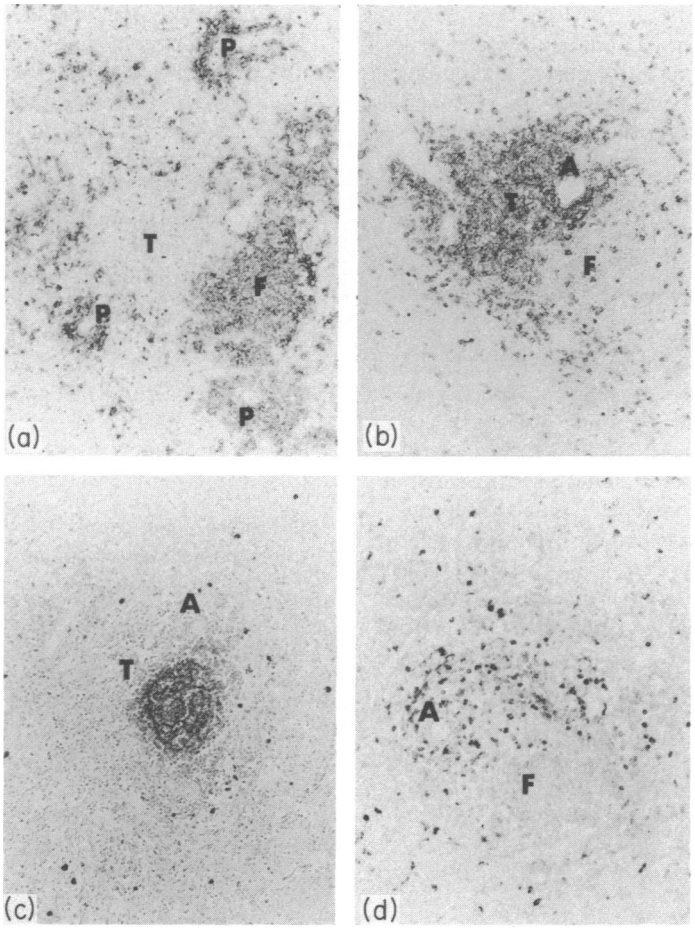

Figure 3. Spleen of 26 g.w. fetus. The basic structure of the white pulp is achieved at this stage. (a) Staining with B1 antibody shows perivascular accumulations (P) at the peripheral area and formation of follicles (F). PALS is also evident (T). (b) T cells stained with Tp40 antibody form PALS (T) around central arterioles (A). Many T cells are also observed in red pulp area. (c) Staining with DRC1 antibody shows mesh-like structures formed by FDC within the follicle (see Fig. 4). (d) S-100 protein-positive cells are evident in PALS. Both round and irregular-shaped cells are observed (see Fig. 5). (Magnification × 80.)

Table 2. Expression of T-cell antigens on fetal thymocytes

	Gestational age (weeks)*						
Antigen phenotype	8–9 ($n=4$†)	10–11 ($n=3$)	12–13 ($n=3$)	14–15 ($n=3$)	16–17 ($n=4$)	18–20 ($n=5$)	Infant‡ ($n=3$)
9.6	45±9§	88±1	93±2	96±1	91±3	95±3	98±2
OKT3	ND¶	48±1	59±1	21±1	41±7	42±5	42±3
OKT4	ND	ND	79±2	81±4	83±2	90±8	78±6
OKT6	ND	60±2	83±4	90±5	86±6	92±6	92±7
OKT8	ND	ND	67±2	76±4	84±3	90±2	86±9
Tp40	ND	90±4	97±5	ND	82±8	94±2	94±4
Tp120	ND	ND	ND	ND	ND	52±10	48±6

* Gestational age (weeks) was determined by post-mortem foot length measurements as correlated with last menstrual period. Values indicate mean ± SEM of the percentage positive cells determined by membrane immunofluorescence assay.

† Number of samples tested.

‡ The ages of the three infants were 6, 8 and 11 months old, respectively.

§ Percentage positive cells determined by membrane immunofluorescence assay.

¶ ND, not determined.

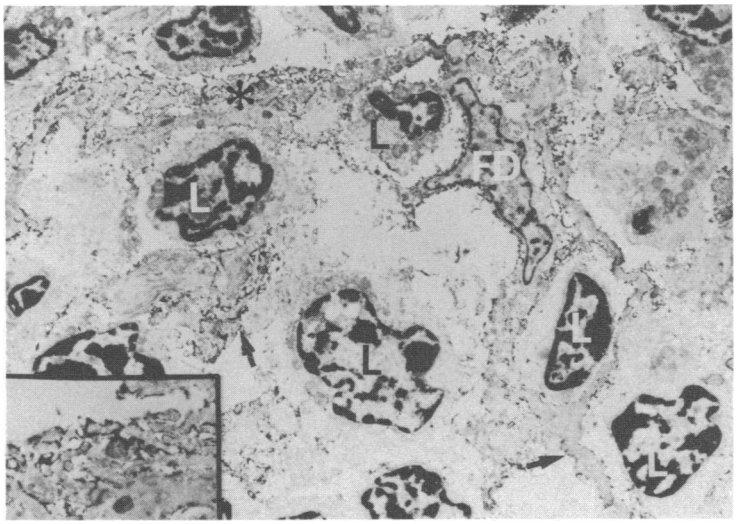

Figure 4. Immunoelectron microscopy of FDC stained with DRC1 antibody (spleen of 26 g.w. fetus). Reaction products are recognized on the surface of FDC (FD) with an irregular nucleus and long cytoplasmic branches (arrow head). Complicated labyrinthine membrane– membrane contacts between FDC are observed (* and inset). No staining is noted on the surface of the adjacent lymphocytes (L) (see Fig. 3c). (Magnification ×3200; inset ×5600.)

by DRC1 antibody (Naiem *et al.*, 1983) were first observed in the 26 g.w. fetal spleen as a clear mesh-like structure within the follicles, concurrently with the formation of the follicles (Fig. 3c). The number and the size of follicles with FDC increased in the succeeding weeks, but there was also a large number of perivascular B-cell accumulations without FDC.

Ultrastructurally, FDC, which had the reaction products of DAB on the surface membrane, were characterized by an irregular-shaped nucleus rich in euchromatin, and by long branching cytoplasmic processes extending between the lymphocytes (Fig. 4). In addition, processes of adjacent FDC were connected to each other with interdigitation.

In contrast to the late appearance of FDC, a small number of IDC, which were detected as S-100 protein-positive cells, appeared around the vessels without being accompanied by T cells at 14 g.w. After 18 g.w., when T cells formed accumulations around arterioles, many IDC were observed predominantly in T-cell areas, constituting PALS (Fig. 3d). Thus, IDC appeared around the vessels 4 weeks prior to the accumulation of T cells. In the early stage of development, the S-100 protein-positive cells were rather small, having round or cerebriform nuclei. However, in the late stage, there appeared large and elongated cells with an irregular nucleus in addition to the small round cells.

Immunoelectron microscopic examination of S-100 protein-positive cells showed electron-dense immunoreaction products distributed diffusely in the cytoplasm. They could be classified into two groups of cells. The first consisted of round cells with a

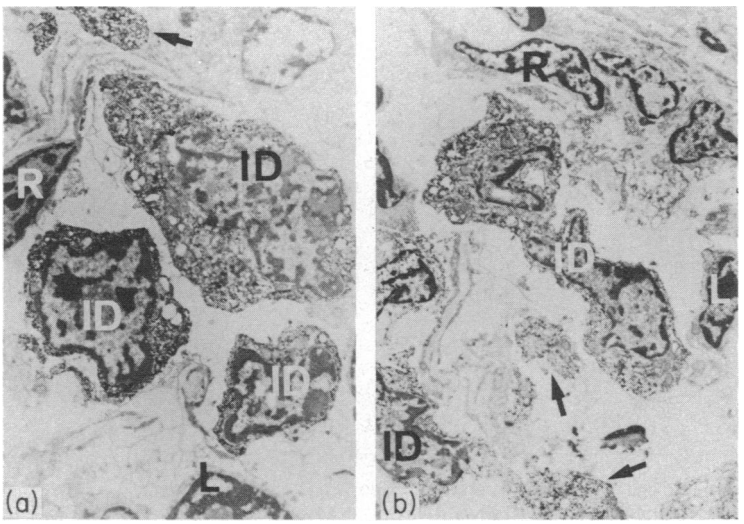

Figure 5. Immunoelectron microscopy of S-100 protein-positive cells (spleen of 26 g.w. fetus). Electron-dense immunoreaction products are observed diffusely in IDC (ID) cytoplasm. Their cytoplasmic extensions are also stained (arrow head). No staining is observed in lymphocytes (L) or reticular cells (R). (a) Two IDC with lymphocyte-like appearance (lower half) and an IDC in transitional form (upper half) are shown. (b) Mature IDC with an irregular nucleus (middle and lower left) are shown (see Fig. 3d). (Magnification ×3200.)

lymphocyte-like appearance (Fig. 5a). The nuclei were round with dense and dark heterochromatin and a prominent nucleolus. The cytoplasm was narrow and contained a small number of organelles. The second group of cells was larger in size, and was irregular in shape and had an irregular nucleus with condensed heterochromatin at the nuclear margin. The cytoplasm was broader, had several extensions, and contained mitochondria and several short profiles of endoplasmic reticulum (Fig. 5b). The first type of cells was predominant in the early stage of development, while the second type increased in number with time, followed by the appearance of typical IDC.

Membrane immunofluorescence analysis of suspension cells

Cell suspensions were prepared from fetal thymus, liver and bone marrow and stained with monoclonal antibodies to compare with the results of immunohistological study. Cells prepared from fetal liver younger than 13 g.w. contained 70–80% erythroid cells, which diminished thereafter concurrently with the increase in blastoid cells and lymphoid cells in reciprocal proportion (Table 3). In contrast, fetal marrow cells contained a small proportion of erythroid cells from 14 g.w. (Table 4).

Fetal thymocytes at 8–32 g.w. were examined for the expression of T-cell antigens (Table 2). As early as 10 g.w., the surface phenotype of fetal thymocytes was already similar to that of infant thymocytes. In fetal liver (Table 3), a very small population (1–3%) of 9·6 antigen-positive T cells also appeared at 9–13 g.w. As for B lymphocytes, a few B1-positive cells, which lack sIg and presumably consist of a pre-B-cell population, were already found in 8–11 g.w. fetal liver; these increased gradually thereafter up to 15–20%. Five to 10% of B1-positive cells were also found in 14 g.w. fetal marrow (Table 4), which was the earliest specimen available for examination. Although B lymphocytes with sIgM were observed in 12–13 g.w., sIgM, sIgD positive B cells were seldom observed in fetal liver and bone marrow throughout fetal life, in contrast to the above described results of immunohistological study of fetal spleen.

DISCUSSION

Immunohistological analysis of developing human white pulp showed that, at the earliest stage, the primitive white pulp was composed of virtually only B1-positive B cells as perivascular accumulations and was the predominant structure until the beginning of T-cell organization at 18 g.w. These T cells positive with 9·6, Tp40 and Tp120 antibodies appeared as small clusters around arterioles centrally to the B-cell areas and progressed rapidly to form the structure of PALS. The organizational process of B and T lymphocytes in the developing human spleen presented here is well correlated to that of mouse system reported by Friedberg & Weissman (1974) who found that the spleen of newborn mouse contained only a few T cells, while prominent accumulations of Ig-positive cells surrounding conspicuous vessels were already observed. These results together suggested that, in the developing white pulp, the earlier organization of B cell might be a general phenomenon in mammals.

One of the interesting findings obtained is that a large number of B cells constituting the perivascular accumulations at an early stage (at 14 g.w.) already had both sIgM and sIgD, whereas this type of B cell was seldom found in any other lymphoid tissues examined at this stage. This result suggested

Table 3. Surface phenotype and cell composition of fetal liver cells

	Gestational age (weeks)*							
	8 (*n*=4†)	9 (*n*=4)	10–11 (*n*=4)	12–13 (*n*=4)	14–15 (*n*=5)	16–17 (*n*=4)	18–19 (*n*=4)	23 (*n*=1)	27 (*n*=1)
Cell composition									
Blastoid	13±3‡	10±2	14±2	22±10	33±8	12±1	36±7	22	8
Lymphoid	8±2	8±1	8±1	11±5	27±2	33±3	33±5	39	35
Erythroid	78±8	80±5	76±1	63±20	25±3	35±7	19±2	26	6
Myeloid	1±1	2±1	2±1	4±4	15±6	20±3	12±3	8	51
Antigen phenotype									
9.6	1>§	2±1	2±1	2±1	3±2	5±3	5±2	6	8
Tp40	1>	3±1	3±1	7±2	5±1	4±1	6±3	10	ND
B1	1±1	2±1	3±1	4±1	10±5	9±7	15±2	12	20
sIgM	0	0	0	3±1	3±1	2±1	13±4	7	12
sIgD	ND¶	0	0	1>	1>	1>	3±1	1	3
NL-12	3±1	4±1	9±3	18±8	22±10	24±9	33±15	32	41
HL-1	4±1	5±1	10±4	12±4	19±5	7±3	14±5	4	35

* Gestational age (weeks) was determined by post-mortem foot length measurements as correlated with last menstrual period. Values indicate mean ± SEM of the percentage positive cells determined by membrane immunofluorescence assay.

† Number of samples tested.

‡ Percentage cell composition determined by May–Grünwald–Giemsa staining.

§ Percentage positive cells determined by membrane immunofluorescence assay.

¶ ND, not determined.

Table 4. Surface phenotype and cell composition of fetal bone marrow cells

	Gestational age (weeks)*						
	14–15 (n=4†)	16–17 (n=4)	18–20 (n=4)	23 (n=1)	26 (n=1)	27 (n=1)	Child‡ (n=3)
Cell composition							
Blastoid	33±5§	32±4	20±16	10	45	16	0
Lymphoid	22±6	29±10	32±8	41	23	45	35±5
Erythroid	15±3	14±1	26±13	16	5	15	17±3
Myeloid	30±6	25±6	22±10	33	27	24	48±11
Antigen phenotype							
9.6	3±1¶	4±2	2±1	6	2	2	22±4
Tp40	3±1	2±1	1±1	2	4	ND**	16±8
B1	8±3	7±2	20±3	17	10	32	12±2
sIgM	3±1	3±1	8±3	13	5	4	5±1
sIgD	1>	1±1	2±1	1	0	0	3±1
NL-12	34±10	42±15	57±10	61	89	60	36±2
HL-1	50±15	51±11	39±14	33	17	28	47±15

* Gestational age (weeks) was determined by post-mortem foot length measurements as correlated with last menstrual period. Values indicate mean ± SEM of the percentage positive cells determined by membrane immunofluorescence assay.

† Number of samples tested.

‡ The ages of the three children were 7, 8 and 11 years, respectively.

§ Percentage cell composition determined by May–Grünwald–Giemsa staining.

¶ Percentage positive cells determined by membrane immunofluorescence assay.

** ND, not determined.

that fetal white pulp provides a microenvironment for differentiation of B1-positive, sIg-negative B cells already found in the 8–11 g.w. fetal liver or for the homing of this type of B cell from liver or bone marrow. DRC1-positive FDC, however, are probably not involved in this early development of B cells because they only appeared much later, at 26 g.w., with the formation of the follicles. The present investigation also suggested that the first structure of B cells, perivascular accumulations, seems to develop histologically into the marginal zone during ontogeny. Although the surface phenotype of B cells in the marginal zone in man was reported to be strongly sIgM and weakly sIgD-positive (Stein *et al.*, 1980), our preliminary study showed that a considerable proportion of these B cells in adult spleen were sIgM- and sIgD-positive, similar to the B cells of perivascular accumulations in fetal spleen. Dijkstra & Döpp (1983) also reported that, in the rat, the first B-cell compartment developed into the marginal zone distinctly from follicles.

In spite of the early appearance of T-cell phenotype and formation of thymic architecture similar to that of infant thymus revealed in suspension cell analysis as well as in immunohistological analysis (R. Namikawa *et al.*, unpublished observations), the homing of T cells to spleen was only noted in fetuses of over 18 g.w. The possibility remains that the thymocytes at an early stage of ontogeny are not sufficiently mature to generate peripheral T cells, or are different from the adult type of thymocytes. Of interest in this connection, Bodger *et al.* (1983) showed that human thymus was first populated by the terminal deoxynucleotidyl transferase (TdT)-negative T lymphocytes, and that a second 'wave' of TdT-positive prothymocytes occurred at about 20 g.w.

In contrast to the late appearance of FDC, the present study showed that IDC detected by anti-S-100 protein antibody appeared 4 weeks prior to the formation of T-cell accumulations. Fetal spleen at 14 g.w. already contained S-100 protein-positive IDC; then, when T-cell areas became well-defined at a late stage, these cells were noted between T cells in the PALS. Wilders, Sminia & Janse (1983) observed the early appearance of Ia-positive dendritic cells, which were considered as IDC, prior to the formation of T-cell areas in Peyer's patches of fetal rat. Dijkstra & Döpp (1983) also reported a similar finding. Together with their results, our present investigation may indicate that IDC provide a suitable microenvironment for the homing of T lymphocytes. Immunoelectron microscopic examination of S-100 protein-positive cells showed cytoplasmic staining of both round, lymphocyte-like cells and cells with cytoplasmic extensions and an irregular nucleus. An obvious transition in form from lymphocyte-like cells to mature IDC was found in the spleens of older fetuses. No lymphocyte-like S-100 protein expressing cells were observed in the adult spleen, so these round, lymphocyte-like cells may be the precursors of IDC.

The present investigation demonstrated the importance of studying lymphoid and non-lymphoid cells with both immunoperoxidase staining and membrane immunofluorescence in order to understand the morphogenesis of splenic white pulp, in relation to the development of the immune system. In conclu-

sion, white pulp may well be an important site for the development of B cells in the early stages, but FDC are not involved in this process. B cells in the marginal zone and follicles may originate from different subpopulations, and IDC play an essential role in the homing of T cells.

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